PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **330th Meeting** *of the Biochemical Society was held in the Department of Biochemistry, University of Cambridge, on Saturday,* 19 *June* 1954, *at* 11 *a.m., when the following papers were read:*

COMMUNICATIONS

An Oxido-reduction Reaction of α-oxoglutarate Coupled with Direct Transfer of an Amino Group.

By F. A. Holton. (*Molteno Institute, University of Cambridge*)

α-Oxoglutarate is known to undergo two distinct oxido-reduction reactions in biological systems:

(*a*) 2α-Oxoglutarate → succinate + CO_2 + α-hydroxyglutarate (Weil-Malherbe, 1937).

(*b*) 2α-Oxoglutarate + ammonia → succinate + CO_2 + glutamate (Krebs & Cohen, 1939).

This communication describes an oxido-reduction which resembles (*a*) in not requiring ammonia but resembles (*b*) in yielding glutamate as the reduced product.

As already briefly reported (Slater & Holton, 1952, 1954), heart muscle sarcosomes (large granules, mitochondria) and crude preparations of yeast hexokinase together bring about an anaerobic disappearance of α-oxoglutarate in the absence of free ammonia, and the reaction is accompanied by phosphorylation. The following additional information has now been gained about this process: (1) The factor in yeast preparations which promotes the reaction is heat-stable and dialysable. Its action cannot be simulated by catalytic amounts of any of the following substances: diphosphopyridine nucleotide, triphosphopyridine nucleotide, diphosphothiamine, pyridoxal phosphate, coenzyme *A*, DL-6-thioctic acid. (2) The disappearance of two molecules of α-oxoglutarate results in the appearance of one molecule each of glutamate, succinate and carbon dioxide. (3) The yeast preparations did not yield ammonia on boiling or during incubation with sarcosomes. (4) The reaction proceeds simultaneously and almost independently of reaction (*b*), which occurs slowly in the presence of large amounts of ammonia.

These results suggest that the reaction studied consists of a dismutation of α-oxoglutarate in which an amino group is transferred directly from some compound in the yeast extract to the oxido-reduction system. The overall process could be expressed by equation (*c*), where Y-NH_2 represents an unidentified amino compound present in the extract.

(*c*) 2α-Oxoglutarate + Y-NH_2 → succinate + CO_2 + glutamate + Y-OH.

This type of reaction, in which an amino donor is converted to a hydroxy compound, instead of a keto compound as in the classical transamination reaction, has been demonstrated explicitly in an anaerobic reaction between L-aspartate and α-oxoglutarate catalysed by sarcosomes. L-Malate and not oxaloacetate was a major product. None of the following amino compounds reacted with α-oxoglutarate under similar conditions: glycine, DL-alanine, L-valine, L-leucine, DL-serine, D-aspartic acid, L-asparagine.

The detailed mechanism of reaction (*c*) may involve a hydrolytic transaminase, or a coupled reaction between a typical transaminase and a dehydrogenase. This coupling would be similar to that suggested by Braunstein (1947) for the mode of action of L-amino acid oxidase.

This work was carried out on behalf of the Agricultural Research Council. My thanks are due to Dr E. L. R. Stokstad of Lederle Laboratories for a gift of DL-6-Thioctic acid.

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Mechanism of Inhibition of Succinic Dehydrogenase by Fluoride and Phosphate.

By E. C. Slater. (*Molteno Institute, University of Cambridge*)

Bonner (1951) showed that inhibition of the succinic oxidase system by fluoride occurred only in the presence of inorganic phosphate. In a detailed study of this inhibition, it was found that both fluoride and phosphate separately, but especially the two together, inhibited succinic dehydrogenase competitively with respect to succinate (Slater & Bonner, 1952). The mechanism of the inhibition was described by equations (1) to (3) (E = succinic dehydrogenase, P = phosphate,

F = fluoride), and the various inhibition constants were calculated.

$$E+P \rightleftharpoons EP, \quad (1)$$
$$E+F \rightleftharpoons EF, \quad (2)$$
$$EP+F \rightleftharpoons EPF \rightleftharpoons EF+P. \quad (3)$$

An alternative mechanism which would fit the kinetic measurements just as well is described by equations (4) and (5):

$$P+F \rightleftharpoons PF, \quad (4)$$
$$E+PF \rightleftharpoons EPF. \quad (5)$$

A choice between the two mechanisms can be made by comparing the inhibitory action of monofluorophosphate with that of fluoride + phosphate. The rates of O_2 uptake (μl./hr.) with various additions were as follows (the figures in brackets are the percentages of inhibition): no addition, 112; + phosphate (0·1 M), 81 (28 %); + fluoride (0·01 M), 103 (8 %); + phosphate + fluoride, 40 (64 %); + fluorophosphate (0·01 M), 86 (23 %). All flasks contained phosphate, 0·002 M; tris(hydroxymethyl)-aminomethane, pH 7·4, 0·06 M; sodium succinate, 0·024 M; ethylenediaminetetraacetate, 0·001 M; heart-muscle preparation, 0·02 ml.; reaction vol., 1 ml.; temperature, 25°.

It is clear that fluorophosphate is much less inhibitory than fluoride + phosphate, in agreement with the mechanism postulated previously.

This work was carried out on behalf of the Agricultural Research Council. I am indebted to Dr Wayne E. White, Ozark-Mahoning Co., Tulsa 1, Oklahoma, U.S.A., for a gift of disodium monofluorophosphate.

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The Resynthesis of Glycogen by Guinea Pig Cerebral Cortex Slices. By F. N. LEBARON.* (*Department of Biochemistry, Institute of Psychiatry, British Postgraduate Medical Federation, Maudsley Hospital, London, S.E.* 5)

Brain glycogen has been shown to fall rapidly to a negligible level after death (Kerr, 1936; Chance & Yaxley, 1950). Using the experimental conditions previously found suitable for resynthesis of labile phosphates (McIlwain & Gore, 1951), it has been demonstrated that glycogen is also resynthesized by guinea pig cerebral cortex slices.

Tissue slices were prepared and incubated by methods previously described (McIlwain & Gore, 1951). Variation of the time between death and final equilibration from 10 to 30 min. did not significantly affect glycogen resynthesis. Respiration was measured in Warburg apparatus during incubation. Experiments were terminated by transferring slices immediately into ethanolic KOH, and glycogen was determined by a micromodification of Kerr's method (Kerr, 1936). This consisted of dissolving the tissue in hot alcoholic KOH, extracting cerebrosides from the residue with a chloroform–methanol mixture, hydrolysing the residual solid for 2 hr. in N-H_2SO_4, and determining reducing substances by a micromodification of Nelson's colorimetric method (Nelson, 1944). By this means the following amounts of glycogen expressed as glucose equivalents were found: slices not incubated, 0·4 μmole/g. fresh tissue; incubated 1 hr., 1·1 μmole/g. fresh tissue; incubated 2 hr., 2·3 μmole/g. fresh tissue; incubated 4 hr., 3·0 μmole/g. fresh tissue. There is no appreciable increase between 4 and 6 hr. The variation between animals is about ± 30 %. Transfer to ethanolic KOH at termination of each experiment required 5–10 sec. No decrease occurred if transfer was delayed up to 1 min. or more. The following facts have been demonstrated as evidence that the substance synthesized is, in fact, glycogen: (1) A red-purple colour, similar to that given by glycogen is obtained with dilute Lugol's iodine solution from the substance isolated as glycogen, but from incubated slices only. (2) This substance gives colour with anthrone (Kahan, 1953) before hydrolysis, as do polysaccharides. (3) This substance does not have reducing action as measured by Nelson's method (Nelson, 1944) before hydrolysis, but the reducing substance obtained on hydrolysis is at least 90 % fermentable by yeast.

Variations in and additions to the incubating medium have shown that glycogen synthesis proceeds equally well in Krebs-Ringer with phosphate buffer or in phosphate-free medium with 'THAM' buffer. Addition of insulin to the normal Krebs-Ringer phosphate to the amount of 0·3 unit/ml. has no effect on the rate or amount of glycogen synthesis. A graded inhibition of synthesis with increasing concentrations of 2:4-dinitrophenol is obtained.

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γ-Methylene-α-Oxoglutaric acid: a Constituent of Groundnut Plants (*Arachis hypogaea*).

By L. Fowden and J. A. Webb. (*Department of Botany, University College, London, W.C.* 1)

Following the isolation of γ-methyleneglutamic acid (MGA) from groundnut plants (Done & Fowden, 1952), evidence has now been obtained that the keto-acid analogue, γ-methylene-α-oxoglutaric acid (MOG) is a normal constituent of the plants. Towers & Steward (1954) have obtained evidence indicating its presence in tulips.

The keto-acids present in 6-day-old groundnut seedlings were investigated by paper chromatography of their 2:4-dinitrophenylhydrazine (DNPH) derivatives using *n*-butanol saturated with 6% (w/v) NH_3 solution as developing solvent. In addition to spots corresponding to the derivatives of α-oxoglutaric (OG), pyruvic and glyoxylic acids, an additional spot, moving just in advance of that of OG, was present on chromatograms. The position of this spot indicated that it was probably due to a dicarboxylic keto-acid of higher mol. wt. than OG. It was therefore co-chromatographed with the DNPH derivative of MOG prepared enzymically by transamination from MGA and OG (following Fowden & Done, 1953); no separation between the two substances occurred during a 40 hr. development.

A larger scale isolation of the DNPH derivative of the new keto-acid was made from one kg. fresh weight of seedlings, using a column packed with Whatman cellulose powder to separate the hydrazones. About 7 mg. of the derivative resulted. 4 mg. of the DNPH derivative of MOG was obtained from a larger-scale transamination, the derivative of unchanged OG again being separated on cellulose powder. The isolated and biologically synthesized products were shown to have similar absorption spectra when dissolved in N-NaOH (differing from that of the OG derivative), and to be essentially identical by X-ray powder photography of their ammonium salts. On hydrogenation (Towers, Thompson & Steward, 1954), both yielded an amino acid inseparable chromatographically from γ-methylglutamic acid in five developing solvents. Chemical synthesis would facilitate an absolute characterization of the isolated material.

Three additional unknown keto-acids, all moving more slowly than OG when chromatographed as their DNPH derivatives, have been encountered in the various groundnut tissue extracts examined.

We wish to thank Prof. K. Lonsdale, F.R.S., for X-ray comparisons and Prof. F. C. Steward for supplying papers in manuscript form. One of us (J. A. W.) wishes to thank the Agricultural Research Council for a personal maintenance grant.

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The Silver Thiol Electrode and Ion-exchange Reactions on the Electrode Surface.

By R. Cecil. (*Department of Biochemistry, University of Oxford*)

During recent work on the potentiometric titration of thiols with silver nitrate (Cecil & McPhee, to be published), it was found that, in neutral or alkaline solution, the silver electrodes acquired a reversibility to thiol. This suggested that the thiols react to form a layer of silver mercaptide on the electrode surface. The production of silver thiol electrodes in this way depends on rather critical silver-plating conditions, and a better method is to dip the freshly plated electrodes in aqueous H_2S for a few seconds and allow the Ag/Ag_2S electrodes thus formed to 'exchange' with the thiol.

The occurrence of ion-exchange reactions on the surface of silver electrodes was confirmed by experiments with other anions. Thus starting with Ag/AgCl, electrodes were made which were in turn reversible to Br^-, CNS^-, CrO_4^{2-}, CO_3^{2-}, I^-. In the same way Ag/Ag_2S electrodes, prepared as described, were made reversible to I^-, Br^- and finally Cl^-. All these electrodes are, of course, reversible to Ag^+.

The reaction at the electrode surface can be written alternatively:

$$RSH + Ag \rightleftharpoons RSAg + H^+ + e,$$
$$RS^- + Ag \rightleftharpoons RSAg + e.$$

One expression of the potential is

$$E = E_0 - \frac{RT}{F} \ln RSH + \frac{RT}{F} \ln H^+,$$

where RSH represents the concentration of unionized thiol. Since this depends on the thiol pK and on pH, measurement of E can give information about the ionization of the thiol group and shows that it ionises before the amino group.

When Dixon & Quastel (1923) and later Michaelis & Flexner (1928) investigated the oxidation-reduction potential of the cystine-cysteine system, they

found that the potentials they obtained bore the same relationship to thiol and hydrogen-ion concentration. This suggests that their electrodes, of Pt, Au and Hg, had reacted with the cysteine and were not inert (see also Freedman & Corwin, 1949).

Detailed studies have so far been made with electrodes reversible to thioglycollic acid, cysteine and homocysteine. The thioglycollic electrodes behave as predicted, but the cysteine, and to a lesser extent the homocysteine, electrodes show anomalous responses at high pH. These correspond approximately to the transfer of half an electron per molecule of cysteine. The explanation is still being sought, but is likely to be some form of association between cysteine molecules.

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The Occurrence of a Methylsulphonium Derivative of Methionine (α-Aminodimethyl-γ-butyrothetin) in Asparagus. By F. CHALLENGER and BARBARA J. HAYWARD. (*Department of Organic Chemistry, The University, Leeds* 2)

The isolation of dimethyl-β-propiothetin chloride $Me_2S^+(Cl^-).CH_2.CH_2.COOH$ from *Polysiphonia fastigiata* by Challenger & Simpson (1948) of the corresponding picrate from *Enteromorpha intestinalis* (Bywood & Challenger, 1953) and of the platinichloride from *Spongomorpha arcta* (Bywood, 1953), established the natural occurrence of sulphonium compounds. An unidentified dimethylsulphonium compound (or compounds) was detected in asparagus (Bywood, Challenger, Leaver & Whitaker, 1951). Jansen (1948) isolated 1:3-dithio*iso*butyric $HS.CH_2.CH(COOH).CH_2.SH$ as the disulphide from asparagus; it did not produce methanethiol in the urine after ingestion.

The sulphonium salt in asparagus differed from the algal thetin; alkaline decomposition of asparagus extracts to dimethyl sulphide required long boiling while the seaweed thetin was readily decomposed by alkali.

An ethanolic extract of asparagus tips was evaporated at 40° under diminished pressure, the residue dissolved in water and, after removal of protein and chlorophyll, applied to a column of sulphonated cross-linked polystyrene (PSX). The retained sulphonium compound was then eluted with ammonia, together with basic amino acids and the eluate applied to 'Deacidite FF' to retain amino acids. The sulphonium effluent, when concentrated, gave a chloroplatinate m.p. 190–193° which evolved dimethyl sulphide with boiling alkali. It had mixed m.p. 196° with the synthetic chloroplatinate m.p. 201°. Both chloroplatinates, after decomposition with metallic silver by the method of Dudley (1929), gave picrates m.p. 158° (synthetic) and 153° (asparagus). The mixed m.p. was 156°. Further purification of the small amount available was impossible. The picrate of the asparagus product dissolved in hydrochloric acid was repeatedly extracted with ether and the resulting chloride solution concentrated. Paper chromatography (butanol–acetic acid–water–ninhydrin) gave a spot corresponding to that obtained with authentic methylmethioninesulphonium iodide and a very faint spot, possibly due to lysine. The solution was then boiled with alkali until evolution of dimethyl sulphide ceased.

The authentic sulphonium iodide was similarly decomposed. Both solutions were passed through PSX, eluted with ammonia and concentrated. Paper chromatography as before gave two pairs of similar spots corresponding to methionine sulphoxide (arising by oxidation of methionine) and homoserine. Asparagus would therefore appear to contain a salt of α-aminodimethyl-γ-butyrothetin, $Me_2S^+(X^-).CH_2.CH_2.CH(NH_2).COOH$.

McRorie *et al.* (1954) isolated methylmethioninesulphonium bromide from cabbage and detected it in parsley, turnip greens, turnips, pepper, onion and lettuce; aqueous hydrolyses of the natural and synthetic products gave homoserine. Formation of dimethyl sulphide is also mentioned.

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The Alkaline Degradation of the Human Blood-group Substances. By K. W. KNOX and W. T. J. MORGAN. (*Lister Institute, London, S.W.* 1)

Treatment of the human *A*, *B* and *H* blood-group mucoids with dilute Na_2CO_3 (pH 10·8 at 100°) results in decrease in viscosity, loss of serological activity, formation of a chromogen from some of the *N*-acetylhexosamine constituents and to complete disintegration of the mucoid (Morgan, 1946). Dialysis of the alkali-treated substances yields a diffusate which gives an immediate colour with Ehrlich's reagent (Aminoff, Morgan & Watkins, 1952), whereas any indiffusible material remaining fails to give this colour reaction.

More gentle treatment ($BaCO_3$ pH 8·5 at 100°) of *N*-acetylglucosamine gives a maximum colour in about 48 hr. Human group substances give in 8 hr. about 15 % of diffusible material, which shows some serological activity and contains a peptide moiety and mucopolysaccharides. The latter possess all the component sugars (L-fucose, D-galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine) of the original substance. The indiffusible material shows some loss in serological activity and viscosity but otherwise is little changed. To reduce the amount of decomposition which occurs when the split products are subject to prolonged heating at pH 8·5, the material after each 1 hr. period at 100° is dialysed and the diffusible material removed. After 32 hr. heating, 10 % of the mucoid remains indiffusible. This material shows little serological activity but largely retains the composition of the original material. The diffusate is concentrated and fractionated at 50, 65, 80 and 90 % (v/v) levels of ethanol. The addition of ether gives a final fraction. About a third of the diffusible product remains soluble.

The ethanol fractions are of the same general composition as the starting material, but all activity is in the fractions precipitated below 65 % ethanol. The ether-soluble fraction contains a series of oligosaccharides which possess the chromogen. These materials have R_F values in collidine of 0·11, 0·22, 0·34 and 0·53 (R_F, *N*-acetylglucosamine, 0·50; *N*-acetylglucosamine chromogen, 0·78).

Monosaccharides and disaccharides, which possess 1:1, 1:3, 1:4 and 1:6 linkages, were treated at 100° and pH 8·5 and the changes studied.

Chromogen is formed from *N*-acetylglucosamine at pH 5·4 and a maximum Ehrlich colour value, equivalent to 70 % of that obtained after 4 min. heating at pH 10·8, was found after 24 hr. at 100°. The group substances behave similarly but the maximum colour (6 days at 100°) is only 10 % of the value obtained from the material after 12 min. at pH 10·8 and 100°.

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Disulphide Interchange Reactions. By A. P. RYLE and F. SANGER. (*Biochemical Laboratory, University of Cambridge*)

The disulphide interchange reaction first reported by Sanger (1953) has been studied in acid, neutral and slightly alkaline solutions using various model systems, with a view to finding under what conditions the reaction occurs and how it may be prevented during chemical studies on protein structure.

In acid solution the reaction between cystine and *bis*-DNP-cystine was followed spectrophotometrically after removal of the unchanged *bis*-DNP-cystine by ether extraction. The yellow ether-insoluble product formed has been crystallized and identified as *mono-N*-DNP-cystine; it is formed by the following overall reaction:

$$\text{DNP-CyS.SCy-DNP} + \text{CyS.SCy} \rightleftharpoons 2\text{DNP-CyS.SCy}.$$

The rate of the reaction is maximal in HCl stronger than 9 N, and in such acid at 37° the reaction between 10^{-3} M cystine and 10^{-4} M *bis*-DNP-cystine stops in 10 hr., when about 90 % of the *bis*-DNP-cystine has reacted. Half the reaction occurs in about 1·5 hr. In 7 N-HCl the reaction is slow, and in 5 N-HCl negligible. The reaction in 10 N-HCl is markedly inhibited by the addition of cysteine to give a ratio SH:SS = 0·01, and is slower in H_2SO_4 than in HCl.

In a mixture of 10 N-H_2SO_4–glacial acetic acid (3:1 v/v) at 37° very little reaction occurs in 13 days. Conditions such as these have been used to effect partial hydrolysis of fragments of insulin without disulphide interchange occurring (see following abstract).

The reaction in neutral solution proceeds rather more slowly and was studied chiefly with a system of 10^{-3} M *bis*-DNP-cystine and 10^{-3} M cystinyl-*bis*-glycine in 0·017 M phosphate buffer. The peptide was used in place of cystine because of the low solubility of the latter at pH 7. It was found that the reaction was about eight times as rapid when oxygen was excluded than when it was present, that the presence of 10^{-4} M cysteine or glutathione produced a very great acceleration and that *p*-chloromercuribenzoate or *N*-ethyl maleimide caused complete

inhibition. The latter inhibitor has been used in structural studies to inhibit the interchange reaction of cystine peptides in neutral solutions.

These results taken together suggest that the reaction in neutral solution is mediated by RS' ions formed by dissociation of thiols (Bersin & Steudel, 1938), which in turn arise from hydrolytic fission of the disulphide bond (Cecil, 1950).

$$R_1SSR_1 + OH^- \rightleftharpoons R_1S^- + R_1SOH,$$

$$R_2SSR_2 + R_1S^- \rightleftharpoons R_1SSR_2 + R_2S^-, \quad \text{etc.}$$

This conclusion is supported by the finding that the speed of the reaction between *bis*-DNP-cystine and glutathione (disulphide) increases with increasing pH. The reaction in acid solution must proceed by some other mechanism since it is inhibited, not catalysed, by thiols.

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The Disulphide Bridges of Insulin. By F. Sanger, L. F. Smith and Ruth Kitai. (*Department of Biochemistry, University of Cambridge*)

The two polypeptide chains of insulin contained in a molecule of mol. wt. 6000 (Harfenist & Craig, 1952) are joined together by the disulphide bridges of three cystine residues. After breaking these bridges by oxidation, the chains could be separated and their amino acid sequences determined (Sanger & Tuppy, 1951; Sanger & Thompson, 1953). They may be represented as follows:

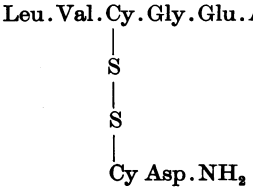
(*A*) Gly^{1} ~ Glu^{5}.Cy^{6}SO$_3$H.Cy^{7}SO$_3$H.Ala^{8}.Ser^{9}.Val^{10}.

Cy^{11}SO$_3$H.Ser^{12} ~ Tyr^{19}.Cy^{20}SO$_3$H.Asp^{21},

(*B*) Phe^{1} ~ His^{5}.Leu^{6}.Cy^{7}SO$_3$H.Gly^{8} ~ Leu^{17}.Val^{18}.

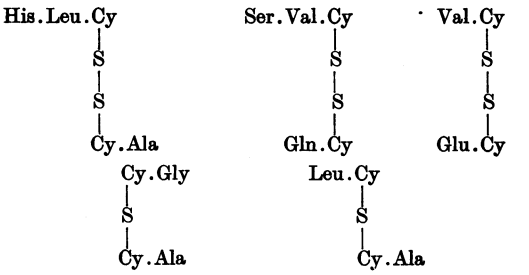
Cy^{19}SO$_3$H.Gly^{20}.Glu^{21}.Arg^{22}.Gly^{23}.Phe^{24} ~ Ala^{30}.

To determine the position of the disulphide bridges, intact insulin was treated with chymotrypsin and the hydrolysate concentrated and brought to pH 5. This yielded a precipitate ('chymotrypsin core') and a solution. The soluble material was fractionated by ionophoresis on paper at pH 6 (Michl, 1951), using the cyanide nitroprusside test (Toennies & Kolb, 1951) to locate the cystine-containing peptides. Each fraction was then oxidized to the corresponding cysteic acid-containing peptides, which were fractionated by ionophoresis, detected by the ninhydrin reaction, eluted and hydrolysed, to identify the amino acids present. One cystine-containing peptide gave rise on oxidation to $CySO_3H.Asp.NH_2(Ac1)$ and Leu.Val.$CySO_3H$.-Gly.Glu.Arg.Gly.Phe.Phe($Bc4$). It therefore has the structure

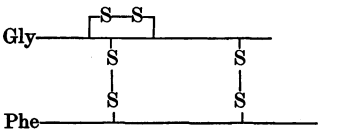


and establishes the presence of a disulphide bridge between positions A 20 and B 19 in insulin. Other similar peptides from this and from a peptic hydrolysate confirmed the presence of this bridge.

The remaining two cystine residues were present in the 'chymotryptic core' and in a similar preparation from a peptic hydrolysate. These cores were subjected to acid hydrolysis under conditions where the 'interchange reaction' is minimal (100 mg. 'core', 5 ml. 20N-H_2SO_4, 3 ml. glacial acetic acid, 2 ml. water, 3 μl. thioglycollic acid. 100°. 30–60 min.). After removal of the H_2SO_4 with an ion-exchange resin, the hydrolysate was fractionated by ionophoresis at pH 6 and 3·6. The cystine-containing bands were eluted, oxidized and subjected to ionophoresis at pH 3·6, to separate the cysteic acid peptides from other non-cysteic acid peptides, and also to some extent from one another. In this manner the following cystine peptides were identified:



These establish that disulphide bridges connect positions A 7 with B 7 and A 6 with A 11, so that the structure of insulin may be represented as follows:



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The Partial Vitamin E Activity of Certain Redox Dyes. By T. MOORE, I. M. SHARMAN and R. J. WARD. (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*)

In previous work (Moore, Sharman & Ward, 1953*a*, *b*, *c*) we followed up reports by Dam, Prange & Sondergaard (1952) that various natural and artificial redox substances, including methylene blue, possess some degree of vitamin E activity. Under our experimental conditions we failed to confirm that methylene blue prevented resorption gestations in deficient rats, or the haemolysis of their erythrocytes by dialuric acid. It was also ineffective in preventing degeneration of the testes. Full activity was found, however, against brown discoloration of the uterus and kidney lesions in rats kept for long periods on diets deficient in vitamin E. Using protection against brown uterus as the criterion we confirmed that thiodiphenylamine was also active, and extended the list to include Bindschedler's green, malachite green, leuco-malachite green and new methylene blue. Several other redox dyes were found inactive at the levels tested. No activity was found for ascorbic acid or cystine, or for any other substance with redox properties but not in the dyestuff class.

In further experiments we have now observed that brown uterus is prevented by rosaniline, as 0·1 % of the diet and by methyl violet (0·126 %). No protection was given by *p*-aminobenzoic acid (0·1 %) or by sulphapyridine (0·1 %). None of these substances was effective in the haemolysis test.

In trials with graded levels of methylene blue, 0·032 % was found sufficient for protection against brown uterus. This level is only one-quarter of the dose (0·126 %) we previously found to be ineffective in preventing resorption gestations or degeneration of the testes. Methylene blue was also tested for its ability to prevent the decline of weight in rats that were beginning to show signs of muscular dystrophy after being kept for nearly a year on the deficient diet. Substantial increases in weight were observed in rats dosed either with the dye or with (±)-α-tocopheryl acetate. The theory that some redox dyes have the anti-dystrophic action of vitamin E, but without its power to protect the reproductive functions, is under investigation.

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The Metabolism of Propionic Acid by Sheep-rumen Epithelial Tissue. By R. J. PENNINGTON and T. M. SUTHERLAND. (*Rowett Research Institute, Bucksburn, Aberdeenshire*)

It has been shown previously that propionic acid is metabolized *in vitro* by sections of sheep-rumen epithelium, and that the rate of uptake of this acid is increased by the presence of carbon dioxide. It was accordingly suggested that propionic acid (or a derivative) may be carboxylated by addition of carbon dioxide. On the basis of other evidence it was considered likely that the carboxylation step did not follow on pyruvate formation (Pennington, 1954).

Propionic acid, when metabolized together with pyruvic acid, completely suppressed the formation of ketone bodies from the latter. The quantity of pyruvate metabolized was correspondingly decreased. In this respect the 'antiketogenic' action of propionate in this ruminant tissue appears to differ from that of glucose, previously described (Pennington & Sutherland, 1953). Propionate similarly suppressed ketone body formation from lactate.

Lactic acid is produced when propionate is metabolized alone. The quantities formed can account for 30–50 % of the propionate disappearing.

When propionate was metabolized in the presence of $^{14}CO_2$ the isotope was incorporated into tissue

metabolites. Approximately one molecule of CO_2 was 'fixed' for each three molecules of propionate metabolized. Most of the isotope was located in the —COOH of the lactate formed. Less than one-tenth as much CO_2 was fixed during the metabolism of pyruvate as when an equivalent quantity of propionate was metabolized.

When $CH_3CH_2{}^{14}COOH$ was metabolized the greater part of the isotope appeared in the carbon dioxide. The specific activity of the lactate formed was only about one-fifth of that of the propionate; this is apparently not due to dilution by endogenously produced lactate because lactate production by the tissue in the absence of substrate is low.

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The Association of Insulin with the Reticulin of Rat Spleen. By J. H. Ottaway. (*Department of Biochemistry, University College, London*)

Chayen & Smith (1954) have shown that insulin labelled with fluorescein becomes bound to the connective tissue of several organs when it is injected into rats. It was of interest to find out whether endogenous insulin is similarly bound to connective tissue fibres.

Reticulin can be conveniently prepared from rat spleen by a method suggested by Dr H. Fell. The spleen is cut freehand into thin slices, which are washed to remove adhering blood, and shaken in Krebs-Ringer phosphate solution at 37° until the tissue has disintegrated. The process may be hastened if necessary by gentle trituration in a mortar. The reticulin fibres are separated by decantation and shaken with successive portions of Ringer solution until the solution is free from haemoglobin. About 50 mg. of moist white fibres, contaminated by only a small number of adhering cells, are obtained from one spleen. The preparation takes 60–90 min., using about 250 ml. of Ringer solution.

In the experiments to be described, spleens were taken from fasted rats which had been injected with heparin to facilitate removal of blood from the tissue. Insulin was detected by the rat diaphragm technique, using the conditions described by Ottaway (1953). The glucose uptake of quarter-diaphragms (weight about 50 mg.) showed a significant increase over the control rate during 45 min. incubation with 10 mg. reticulin from normal spleen. Incubation with reticulin from spleens of alloxan-diabetic rats did not affect the glucose uptake. The solution used for the final (10 min.) washing of the reticulin fibres did not contain any detectable amount of insulin.

It is concluded that the connective tissue of rat spleen (and presumably of other organs also) normally contains insulin which can slowly dissociate in an active form. The relations of this phenomenon to the binding of insulin in tissues described by Stadie and his collaborators (Stadie, 1954), and to the insulin-like effect of growth hormone (Ottaway, 1953), have been studied.

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The Association of Insulin with Reticulin in Rat Tissues. By J. Chayen and R. H. Smith. (*Wheatstone Laboratory, King's College, London, W.C. 2 and School of Biochemistry, University of Cambridge*)

Glucose utilization studies on the isolated rat diaphragm after exposure to insulin led Stadie (1954) to conclude that insulin is bound to certain, unspecified tissue elements. Subsequent experiments (Stadie, 1954) with isotopically labelled insulin supported this conclusion but did not indicate the site at which the insulin was bound.

In an attempt to elucidate the site of action of insulin in isolated tissues, we observed that conjugates of insulin with fluorescent dyes possess a high affinity for the reticulin components of rat diaphragm. The insulin conjugates were prepared with either dimethylaminonaphthalene-5-sulphonyl chloride (Weber, 1952) or fluorescein isocyanate (Kaplan & Coons, 1951); they contained 2 moles of dye per mole of insulin. Insulin thus modified, retained at least 50 % of its original hypoglycaemic activity.

The insulin-binding effect was observed in diaphragms taken from rats injected 2 hr. previously with labelled insulin and in normal diaphragms incubated for periods of 15 to 60 min. with Stadie medium containing labelled insulin. Simple teasing of the fresh, unfixed tissue with fine needles in Stadie medium was found to give the best preparations for fluorescence microscopy. In these teased preparations the characteristic yellow fluorescence of the administered insulin conjugate can readily be seen to be associated with the fine reticulin network of the muscle. Tendon collagen fibres, in contrast to reticulin, do not bind insulin conjugates to an observable extent. The reticulin of spleen and adipose tissue preparations also shows a marked affinity for insulin conjugates.

Controls were carried out with the following substances which were administered 2 hr. before excising the diaphragms: fluorescein, the fluorescein conjugates of whole serum (human) lactoglobulin (cryst.), ribonuclease (cryst.), prolactin and somatotrophin (cryst.). Apart from somatotrophin, which appeared to bind like insulin, none of these substances had an observable affinity for rat diaphragm reticulin.

The reversible nature of the binding of insulin by reticulin is indicated by the fact that the yellow fluorescence of reticulin in diaphragm preparations slowly becomes diffuse: after standing 2–3 hr. in Stadie medium, at room temperature, it is not easy to distinguish the yellow secondary fluorescence from the primary fluorescence of the tissue. The experiments of Ottaway (1954) also provide evidence that this binding effect is reversible.

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Dependence of Sulphate Reduction and Oxygen Utilization on a Cytochrome in *Desulphovibrio*.

By J. R. Postgate (introduced by V. A. Knivett). (*Chemical Research Laboratory, Teddington*)

The sulphate-reducing bacteria are unusual among anaerobes because they contain a thermostable cytochrome (α, 553 mμ.; β, 525 mμ.; γ, 419 mμ.). It may be extracted in solution and concentrated; a soluble green protein absorbing at 630 mμ. has none of the properties of a conventional cytochrome (Postgate, 1954).

Cells incubated *in vacuo* with excess of sulphate or sulphite showed α and β bands of diminished intensity compared with cells without sulphate provided precautions were taken to remove sulphide; the 630-component was unaffected. This phenomenon was temporarily antagonized by structural analogues of sulphate (selenate or monofluorophosphate) suggesting that the cytochrome is directly concerned in sulphate reduction.

The cytochrome bands disappear on shaking a cell suspension in air, and return on standing or passing in H_2 (Postgate, 1954), suggesting that oxidation of substrate with oxygen by these bacteria is in principle possible. Washed suspensions of the Hildenborough strain have now been shown to oxidize lactate or pyruvate slowly in air ($-Q_{O_2}$ 8 mm.^{3}/mg. dry wt./hr.; cf. Q_{CO_2} values of 60–120 with sulphate). If gaseous hydrogen was the substrate, however, a rapid Knallgas reaction occurred, and cells rich in hydrogenase reduced oxygen as readily as they reduced sulphate, though not as fast as they reduced methylene blue. The rate of the Knallgas reaction depended on the oxygen concentration up to 4% (v/v); higher oxygen concentrations progressively inhibited the reaction presumably owing to oxidative destruction of hydrogenase during the experiments.

Cetyltrimethylammonium bromide (100 μg./mg. dry wt. cells) liberated cytochrome into the medium and abolished sulphate reduction without affecting the reduction of methylene blue by hydrogen. Such cells conducted the Knallgas reaction only slowly, but the rate of reaction was augmented by addition of a concentrate of the cytochrome, but not by denatured cytochrome or muscle cytochrome *c*. Hence the rate of the H_2–O_2 reaction is dependent on the cytochrome concentration. Both crude and purified preparations of the cytochrome are autoxidizable, and it is likely that the utilization of oxygen, paradoxical in an anaerobe of so exacting a character, is simply a result of this chemical property.

I am indebted to Dr Keilin for suggesting the primary experiments on oxygen utilization.

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The Hydrolysis of Cortisone Acetate by Enzymes of Human Blood. By F. Hobbiger, Sylvia A. Simpson and J. F. Tait. (*The Middlesex Hospital, Medical School, London, W.* 1)

Although Zeller, Fleisher, McNaughton & Schweppe (1949) have described the hydrolysis of deoxycorticosterone acetate by stromata of human red cells, detailed studies of the processes involved in the hydrolysis of steroid acetates by enzymes of human blood have not been reported. This lack of information is probably due to the difficulty of applying the usual techniques for the study of esterases because of the low solubility of most steroid esters in biological fluids.

A technique has been developed for the measurement of the rate of hydrolysis of cortisone acetate at low concentrations. 30 μg. [4-^{14}C]cortisone acetate in 3 ml. solution were incubated with preparations of human red cells and plasma and purified true and pseudo-cholinesterase under anaerobic conditions at 37° and at a pH of 7. The labelled material was extracted by partition methods and the resulting residue chromatographed in the Bush *B* (3) system (Bush, 1952). The steroid ester and alcohol were located by the method of Haines & Drake (1950) and the radioactivity in the appropriate ultraviolet adsorbing areas was measured by mica-window counting. The ratio of the counts due to the alcohol compared with the ester gave the value for the amount of hydrolysis.

Using this technique the hydrolysis of cortisone acetate by human red cells, which contained the main enzymatic activity of whole blood, was investigated in detail. No difference was found between intact and lysed cells. A comparison of the activity of highly purified true cholinesterase and preparations of red cells, in concentrations equally active against acetylcholine, showed that true cholinesterase was only partly responsible for the hydrolysis of cortisone acetate by human red cells. This indicated that in addition to true cholinesterase another esterase or esterases must be involved. This was confirmed by a study of the effect of potent inhibitors at concentrations which completely abolished cholinesterase activity. Thus, although di*iso*propylphosphorofluoridate and tetraethylpyrophosphate inhibited completely the enzymatic hydrolysis of the steroid ester, most of the activity was not affected by eserine or 3-(diethoxyphosphinyloxy)-*N*-methylquinolinium methylsulphate.

The relation of this esterase to other esterases present in human blood (Mounter & Whittaker, 1953) will be discussed.

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The Incorporation of Halogenated Pyrimidines into Deoxyribonucleic Acids. By D. B. Dunn and J. D. Smith. (*Agricultural Research Council Plant Virus Research Unit, Molteno Institute, Cambridge*)

The growth of *Streptococcus faecalis* R. is inhibited by 5-bromouracil. Weygand, Wacker & Dellweg (1952) demonstrated that after growth in the presence of 5-bromouracil containing the isotope ^{82}Br substantial amounts of labelled 5-bromouracil could be isolated from *S. faecalis* nucleic acids after hydrolysis with $HClO_4$.

When *Bacterium coli* is made thymine-requiring by growth in a medium containing sulphanilamide (Winkler & de Haan, 1948), 5-bromouracil and 5-iodouracil inhibit growth. 2–3 hr. after their addition the bacterial growth rate is decreased and thereafter remains approximately constant. The bacteria grow to their normal maximum density, but the viable count is reduced. The degree of inhibition by 5-bromouracil increases with the ratio 5-bromouracil/thymine in the medium. *Bact. coli* inhibited by 5-bromouracil or 5-iodouracil will support the growth of the bacteriophages T_2r and T_5.

Deoxyribonucleic acids (DNA) from *Bact. coli*, T_2r and T_5 grown in the presence of 5-bromouracil, were hydrolysed in 72% (w/w) aqueous $HClO_4$ (Marshak & Vogel, 1950), and the bases separated by two-dimensional paper chromatography. 5-Bromouracil, identified by paper chromatography, electrophoretic mobility and ultraviolet spectroscopy, was found to replace quantitatively a fraction of the thymine (26% in *Bact. coli* DNA, 77% in T_2r and 30% in T_5 DNA). 5-Iodouracil on hydrolysis in $HClO_4$ in the presence of DNA breaks down quantitatively to uracil. Hydrolysis of DNA from *B. coli* and T_2r grown in the presence of 5-iodouracil gave uracil in amounts corresponding to a replacement by 5-iodouracil of 17% (*Bact. coli*) and 50% (T_2r) of the thymine in the nucleic acid.

After hydrolysis of the deoxyribonucleic acids with deoxyribonuclease and rattlesnake venom diesterase, substances with the expected properties of 5-bromouracil and 5-iodouracil deoxyribonucleo-

tides were isolated. These migrated ahead of the other mononucleotides on paper electrophoresis at pH 9. On hydrolysis in 72 % $HClO_4$, 5-bromouracil deoxyribonucleotide gave equimolar proportions of 5-bromouracil and phosphate, while 5-iodouracil deoxyribonucleotide gave uracil.

Although purified T_2r and T_5 containing 5-bromouracil or 5-iodouracil had the normal ultraviolet absorption spectra, and electron microscopy of the T_2r 'bromouracil' preparations showed largely particles of normal structure, a large proportion of the particles in each of these preparations were non-infective. T_2r preparations in which 77 % of the thymine had been replaced by 5-bromouracil contained about 70 % non-infective particles.

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The Oxidation *in vitro* of Labelled Glucose and Acetate by Slices of Lactating Rat Mammary Tissue. By W. G. DUNCOMBE and R. F. GLASCOCK. (*National Institute for Research in Dairying, University of Reading*)

By a technique exactly similar to that previously described (Duncombe & Glascock, 1953) with sheep tissue the oxidation *in vitro* (bicarbonate-Ringer) of uniformly labelled glucose (0·3 %) and doubly labelled acetate ($^{14}CH_3{}^{13}COONa$; 0·02 M) has now been studied in lactating rat mammary tissue. It oxidizes more carbon, when glucose is sole substrate, than does sheep tissue (rat 5–10, sheep 1–2 μg. C/hr./mg. dry weight tissue). Whereas with sheep mammary tissue acetate increased the oxidation of glucose carbon, in rat mammary tissue acetate has no measurable effect. Pyruvate (0·02 M), however, reduces by about 50 % the oxidation of glucose. This is presumably due to dilution by unlabelled pyruvate of labelled pyruvate derived from glucose via the glycolytic cycle.

Rat mammary tissue oxidizes acetate, as sole substrate, much less actively than does sheep tissue (rat 0·1–0·4, sheep 1–4 μg. C/hr./mg. dry weight tissue) and about 50 % more carboxyl carbon than methyl carbon appears in the CO_2. In 23 experiments (seven animals) the ratio of carboxyl to methyl carbon oxidized to CO_2 was in the range 1·11–1·57 with a mean of 1·42.

Whereas in sheep mammary tissue, glucose increased the amount of acetate carbon oxidized, in rat tissue glucose reduces the amount oxidized by about 50 %, the effect on the two carbon atoms being quantitatively different however. The mean ratio of carboxyl to methyl carbon oxidized then becomes 2·06 with a range of 1·72–2·93; i.e. glucose depresses the amount of methyl carbon oxidized more than it does carboxyl carbon. It would thus appear that the observed effect of glucose on the oxidation of acetate is not due solely to the production of unlabelled C_2 units which dilute those derived from the unlabelled acetate.

It will be noted that the effect of added glucose on the oxidation of acetate by rat mammary tissue is different from that reported by Popjak & Tietz (1954). Using phosphate saline (gas phase = O_2) they found that rat mammary tissue oxidized about 5 times more acetate carbon in the presence of glucose than in its absence. We have repeated their experiment using phosphate saline and have observed the same effect. There is thus a marked difference in the metabolism of the slices in the two media.

Thanks are due to Dr S. J. Folley, F.R.S., for his advice and encouragement in this work, to Dr D. H. Tomlin for carrying out the ^{13}C determinations in the Agricultural Research Council's Mass Spectrometer, to Mr B. W. E. Peaple for technical assistance and to Dr Helen Porter who made the radioactive starch from which the [^{14}C]glucose was prepared.

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Glutathione and Ascorbic Acid in the Metabolism of Germinating Peas. By S. P. SPRAGG and E. W. YEMM. (*Department of Botany and Biological Chemistry Laboratories, University of Bristol*)

Hopkins & Morgan (1943) have shown that reduced glutathione is rapidly formed in pea seeds during the early stages of germination. The activity of the tripeptide in enzymic systems of oxidation reduction (Mapson & Goddard, 1951), transpeptidation (Hanes, Hird & Isherwood, 1952) and glycolysis (Krimsky & Racker, 1952) indicates that it may play an important part in regulating cellular metabolism. This possibility has been explored by experiments in which the changes of glutathione, ascorbic acid and respiratory processes were studied in germinating pea seeds.

Evidence has been obtained that in the first stage of germination, 0–6 hr. in air at 22°, there is a rapid conversion of oxidized (GSSG) to reduced glutathione (GSH). The total amount of GSH + GSSG does not change significantly over the first 24–26 hr. of germination, suggesting that extensive synthesis or breakdown does not occur in these early stages. Ascorbic acid (AA) increases relative to dehydroascorbic acid (DHA) during the first 24 hr., but a loss of total AA + DHA is generally observed. Later both AA and DHA show substantial increases as germination proceeds.

The respiratory activities of the seed over the first 48 hr. of germination can be divided into three phases: (1) A rapid rise in O_2 uptake and CO_2 production during the uptake of water and extending over 6–9 hr. (2) A period from about 9 to 30 hr. in which respiration is maintained at a fairly steady rate. (3) A further increase of rate associated with the rupture of the testa of the seed. Several lines of evidence indicate that the interruption of the rising rate of respiration during phase (2) is in part attributable to the limitation of gaseous diffusion by the testa, and that at this stage a substantial part of the CO_2 arises from anaerobic glycolysis.

The increase in the proportion of the reduced forms of GSH and AA may be related to the restricted supply of oxygen to the tissues and the formation of GSH may play a significant part in the activation of —SH enzymes during the first phase of germination.

This work is part of the programme of the Food Investigation Organisation of the DSIR.

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Estimation of Amino Acids by Ninhydrin. By E. C. Cocking and E. W. Yemm. (*Department of Botany, University of Bristol*)

Moore & Stein (1948) and Smith & Agiza (1951) have described quantitative methods for estimating amino acids colorimetrically by means of the reaction with ninhydrin. Both these methods suffer from the disadvantage that equimolecular amounts of different amino acids do not produce equal colour intensities. More recently Troll & Cannan (1953) have modified the procedure so that a stoichiometric reaction occurs with quantitative yields of dioxohydrindylidene-dioxohydrindamine (DYDA), the probable end product of the reaction. For routine purposes the modified reaction has several disadvantages: it is difficult to control the pH accurately, high water blanks are obtained and it involves extensive use of a phenol–pyridine solvent. It has been possible to retain the advantage of the quantitative reaction of Troll & Cannan with a simple procedure suitable for routine analysis.

The method may be summarized as follows:

1 ml. of an amino acid solution containing 0·05–2·8 μg. amino nitrogen was mixed with 0·5 ml. citrate buffer pH 5 (0·2 M). The ninhydrin in methyl cellosolve (0·2 ml., 5 % w/v) and 0·01 M–KCN in methyl cellosolve (1 ml., 2 % v/v) were added to this solution either separately or as a single solution. Separately these reagents are stable for at least 1 month, and mixed, for at least 1 week. The well-mixed solution was heated for at least 15 min. at 100° and cooled for 5 min. in running tap water. The b.p. of the water–methyl cellosolve mixture is greater than 100°, and using tubes stoppered with a glass marble, evaporation losses during the heating period were negligible. The solution was made up to a convenient volume with ethanol (60 % v/v), and the optical density determined on a Unicam Spectrophotometer at 570 mμ, or the colour intensity read on an EEL colorimeter using filter 626. The colours were quite stable for at least 1 hr. at room temperature. It was found that most of the common amino acids gave colours equivalent to 100 ± 1 % of that of pure DYDA, except tryptophan (80 %) and lysine (110 %). Ammonia reacted yielding a colour equivalent to only 33 % of that of pure DYDA.

The following advantages are claimed for the above procedure:

(1) More complete reaction with amino acids gives greater sensitivity and more reproducible results.

(2) Lower sensitivity to ammonia results in small and consistent blank values.

(3) Shorter and less critical heating period is required.

(4) Considerable economy of ninhydrin: only about half the quantity is required compared with the method of Moore & Stein.

(5) It avoids the complication of storing reduced ninhydrin reagent under nitrogen.

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The Reaction of Disulphides with Sodium Sulphite. By R. CECIL and J. R. MCPHEE. (*Department of Biochemistry, Oxford*)

The reaction kinetics of four disulphide compounds with sodium sulphite have been studied, namely cystine, *NN'*-diacetylcystine diethyl ester, *NN'*-diformylcystine and dithiodipropionic acid. The equations are

$$RSSR + SO_3'' \rightleftharpoons RS^- + RSSO_3^-,$$
$$RS^- + H^+ \rightleftharpoons RSH,$$
$$SO_3'' + H^+ \rightleftharpoons HSO_3^-.$$

Reactions were carried out in buffered solutions at 25°. Samples were acidified with dilute nitric acid to pH ≃ 2, and the thiol estimated by potentiometric titration with silver nitrate (Cecil & McPhee, to be published).

Acetylcystine ester shows the simplest behaviour because it has no ionizing groups. The reaction is found to be of the first order with respect to each reactant. It is reversible (Stricks & Kolthoff, 1951), the rate of the reverse reaction depending on the concentration of RS^-. At pH > 9 this becomes significant, and the sulphite is all in the form SO_3''. By making use of the equilibrium constant under the same conditions, the specific rate constant for the forward reaction with SO_3'' can be calculated. This is found to be independent of pH.

At pH 4·5–7 the reaction is no longer reversible owing to the absence of RS^-, but the sulphite–bisulphite equilibrium has to be taken into account. The reaction mechanism is complex, and only the initial rates are considered. Apparent rate constants are found which decrease with decreasing pH. If both SO_3^{2-} and HSO_3^- react with disulphide then these apparent rate constants could be interpreted in terms of two specific rate constants, the pK_a of sulphurous acid, and the pH. It was found that HSO_3^- does not react with these disulphides, and change in the apparent rate constants with pH can be accounted for by the corresponding change in SO_3^{2-} concentration. The specific rate constant agrees with that found in the higher pH range.

The other compounds show the same general behaviour, complicated only by the presence of charged groups. It is found that an overall negative change on the molecule greatly reduces the specific rate constant. Thus acetyl cystine ester gives $k = 265$ l.mol.$^{-1}$ min.$^{-1}$, whereas diformyl cystine and dithiodipropionic acid give $k = 9$ and $k = 1{\cdot}1$ respectively. 'Neutral' cystine, i.e. in the zwitterion form, gives $k = 1100$, whereas completely ionized cystine gives $k = 3$. Thus at high pH cystine gives apparent rate constants which decrease with increasing pH. The specific rate constants for the different ionic species may be calculated from the apparent constants and the pK's of the amino groups.

One of us (J.R.M.) is indebted to the Commonwealth Government of Australia for a CSIRO traineeship.

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DEMONSTRATIONS

An Apparatus for Determining the Freezing-points of Small Volumes of Fluid. By G. R. HERVEY (introduced by R. A. MCCANCE). (*Medical Research Council Department of Experimental Medicine, University of Cambridge*)

The apparatus uses thermocouples and a potentiometer system to measure the freezing points of volumes of fluid of 0·1 ml. or less. The accuracy is 0·01° and the apparatus is reasonably simple to construct, and rapid and convenient to operate.

Paper Electrophoresis of Lipids and Lipoproteins of Human Sera. By W. G. DANGERFIELD and ELSPETH B. SMITH. (*Department of Pathology, St Bartholomew's Hospital, E.C.* 1)

The lipids on paper electrophoresis strips are stained with Sudan Black in 55 % (v/v) ethanol. This gives two main bands of staining, the α- and β-lipoproteins, each of which consists of two or more components. Further resolution of the bands can be achieved by extracting the unstained strips with organic solvents, which remove a large part of the lipid, particularly the neutral fat and cholesterol; this shows up other less soluble bands more clearly. A particularly characteristic insoluble pre-β-lipid band is found in serum from patients with type II nephritis.

Characteristic lipid patterns are found in a number of pathological conditions, and injection of heparin produces distinctive changes in the patterns.

The Liebermann reaction has been adapted for staining cholesterol on the paper; owing to the rapid uptake of moisture from the atmosphere the colour is not stable, but it lasts long enough to enable the strip to be photographed. Autoradiographs made from the sera of patients treated with ^{32}P show the possible distribution of phospholipid.